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13. ABSTRACT ***(Maximum 200 Words)***

Apoptosis is a cellular suicide program that specifically rids an organism of damaged or superfluous cells. To define early signaling events that are required for the induction of apoptosis, we have utilized a Xenopus egg extract system capable of recapitulating several biochemical hallmarks of apoptosis in vitro. Using this in vitro egg extract system we have previously identified the adaptor protein crk as a necessary component in the apoptotic signaling pathway of these egg extracts. Furthermore, we have established that the removal of crk SH2 or N-terminal SH3 binding proteins from the extract also inhibits apoptosis. Using affinity chromatography, we have identified the major phosphotyrosine containing crk SH2 binding protein present in the egg extracts as Xenopus wee1. Wee1, a tyrosine kinase previously characterized as a cell cycle regulator, specifically binds to the recombinant crk SH2 domain and, furthermore, wee1 and crk can be co-immunoprecipitated from the egg extracts. We have found that addition of wee1 directly to the Xenopus egg extract accelerates apoptosis. In contrast, the addition of specific anti-wee1 antisera directly to the extract inhibits apoptosis. Moreover, wee1 exerts this pro-apoptotic function upstream of the mitochondrial requirement for apoptosis. Finally, adding recombinant Xenopus wee1 protein to an extract previously depleted of crk SH2 binding partners rescues apoptosis. These data represent the first documented direct interaction between wee1 and an SH2 domain containing protein and is the first study to implicate a pro-apoptotic signaling capacity for the wee1 tyrosine kinase.

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Erica K. Evans 12/30/99

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Introduction

Apoptosis is a process which eliminates imperfect or nonessential cells in multicellular organisms. Apoptosis plays a critical role in embryonic development, the maintenance of tissue homeostasis, and immune system regulation; the misregulation of the apoptotic process may result in numerous pathologies including tumorigenesis. Significant advances have been made in understanding the final stages of apoptotic death. For example, several key molecules shown to be involved in the dismantling of an apoptotic cell have been described. These include proteins such as the caspases, CAD (caspase activated DNase), and AIF (apoptosis inducing factor). However, in most cases the upstream molecules that initiate an apoptotic signaling cascade are still unknown. The primary goal of this work is to elucidate early signaling events necessary for the induction of apoptosis.

To investigate upstream signaling in the apoptotic program, we have employed the Xenopus egg extract system which faithfully reproduces the phenotypic and biochemical characteristics of apoptosis. We have previously shown that the crk protein, consisting of one SH2 (Src homology 2) and two SH3 (Src homology 3) domains is required for apoptotic signaling in the Xenopus egg extract. That is, immunodepletion of the crk protein inhibits apoptosis in the egg extract system. Furthermore, depletion of crk SH2 or N-terminal SH3 domain binding partners from the extract also inhibits apoptosis. These data suggest that both crk SH2 and N-terminal SH3 domain ligands are necessary to propagate an apoptotic signal and that the identification of these binding proteins would provide significant insight into the signaling mechanisms responsible for the induction of apoptosis in the egg extracts. To this end, we have shown that the crk SH2 domain specifically binds the tyrosine phosphorylated Xenopus wee1 protein. In addition, we have demonstrated that the wee1 protein will accelerate apoptosis when added to the egg extract and importantly, addition of recombinant wee1 protein will rescue apoptosis in an extract previously depleted of crk SH2 binding proteins.

Body

As described in my Annual Summary submitted September 30, 1998, using a recombinant crk SH2 affinity column in a large scale protein purification protocol and mass spectoscopic sequencing techniques, we have identified the major phosphotyrosine containing crk SH2 domain ligand as the Xenopus wee1 tyrosine kinase. To summarize, western blot analysis has demonstrated that wee1 specifically binds to the recombinant crk SH2 domain and crk full length protein but not a crk SH2 domain mutated to abrogate phosphotyrosine binding. In addition, crk and wee1 are co-immunoprecipitated from the egg extract suggesting these two proteins interact endogenously.

A role for wee1 in apoptotic signaling

To assess wee1's role in apoptosis, we obtained a His tagged wee1 baculovirus stock from Dr. Bill Dunphy. Addition of purified Xenopus wee1 protein to the extract at a 1:10 dilution (final concentration 2ng/ul) accelerates the onset of apoptosis in the extract by 1-1.5 hours by all assays used to measure apoptosis; visual chromosome condensation and nuclear fragmentation, caspase activity, and mitochondrial cytochrome c release (Figure 1). These data suggest that wee1 can positively regulate apoptosis in the Xenopus egg extract system.

To further assess wee1's role in apoptotic signaling, we added polyclonal wee1 antisera directly to the egg extract and assayed its effect on apoptosis. The addition of polyclonal antisera directed against wee1, but not control non-immune antisera, delayed the onset of apoptosis in the extract as measured by caspase activation (Figure 2). These experiments with blocking antisera suggest that disrupting wee1's accessibility to substrates or perhaps blocking its ability to bind other proteins such as crk, inhibits apoptosis in the extract.

Mitochondrial cytochrome c release has been shown to be a critical step in the apoptotic pathway. Release of cytochrome c into the cytoplasm results in the activation of caspases, the proteases responsible for dismantling an apoptotic cell. To determine if wee1 acts upstream of mitochondrial cytochrome c release, recombinant wee1 was added directly to purified cytoplasm

(lacking mitochondria), and caspase activity was assayed (Figure 3). Wee1 addition to purified cytoplasm did not result in caspase activity suggesting wee1, like crk, acts upstream of the mitochondrial requirement in apoptotic signaling.

To determine if the wee1 protein is a relevant crk SH2 binding protein involved in apoptotic signaling, we added recombinant wee1 to a crk SH2 depleted extract and assayed the ability of this extract to accelerate apoptosis. The addition of recombinant wee1 did rescue apoptosis in a crk SH2 ligand depleted extract (Figure 4) suggesting a tyrosine phosphorylated wee1 binds to the SH2 domain of crk to transmit an apoptotic signal. Taken together the data presented in this report demonstrate a pro-apoptotic role for the wee1 tyrosine kinase and support a model in which a tyrosine phosphorylated wee1 protein interacts with the crk protein through the SH2 domain to induce apoptosis in the Xenopus egg extract.

Recommendations in relation to the Statement of Work

Technical Objective 1: Identification of those proteins which participate in apoptotic signaling by binding to the SH2 domain of crk. I have completed tasks 1 through 7 which included large scale purification of proteins bound to the crk SH2 domain and functional characterization of isolated proteins in apoptotic signaling. The data obtained suggest that a tyrosine phosphorylated wee1 protein interacts with the crk adaptor protein to induce apoptosis in the Xenopus egg extract. The anti-SAM 68 Western blot originally proposed in task 1 was omitted as the 65kD phosphotyrosine containing protein which bound to the crk SH2 domain was demonstrated by microsequencing to be wee1.

Technical Objective 2: Identification of proteins which bind to the crk SH3 glutathione sepharose beads in apoptotic extracts. As mentioned in last year's Annual Summary, because the characterization of the crk SH2 domain binding partners has expanded, another member of our lab, Jesse Smith has sought to identify crk SH3 binding proteins relevant for apoptotic signaling.

A protein not previously known to interact with SH3 domains has been revealed and the functional characterization of that protein in apoptotic signaling continues.

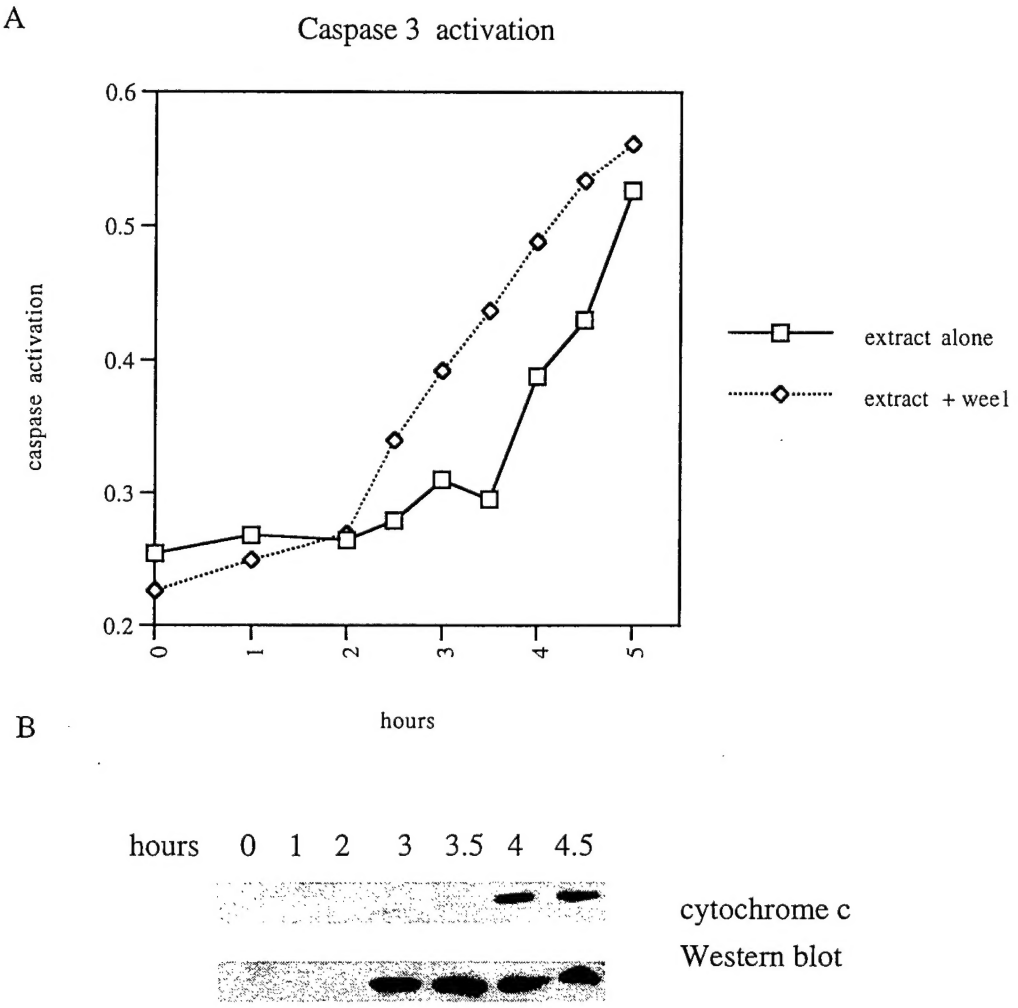

Figure 1. Wee1 addition to Xenopus egg extract accelerates apoptosis. A. Recombinant Xenopus wee1 protein was added to 1:10 dilution to Xenopus egg extracts and the onset of apoptosis was measured by caspase activation. Without wee1 addition the extract demonstrated caspase activity after 4 hours room temperature incubation. However, when wee1 was added to the extract, caspase activity was detected after only 3 hours at room temperature. B. At specific time intervals shown, an aliquot of egg extract was filtered to remove mitochondria and the filtered cytoplasm was assayed for the presence of cytochrome c by Western blot analysis. Cytochrome c release from the mitochondria was detected after only 3 hours at room temperature in extracts which were supplemented with wee1. In contrast, in extracts without additional wee1, mitochondrial cytochrome c release was detected after 4 hours room temperature incubation.

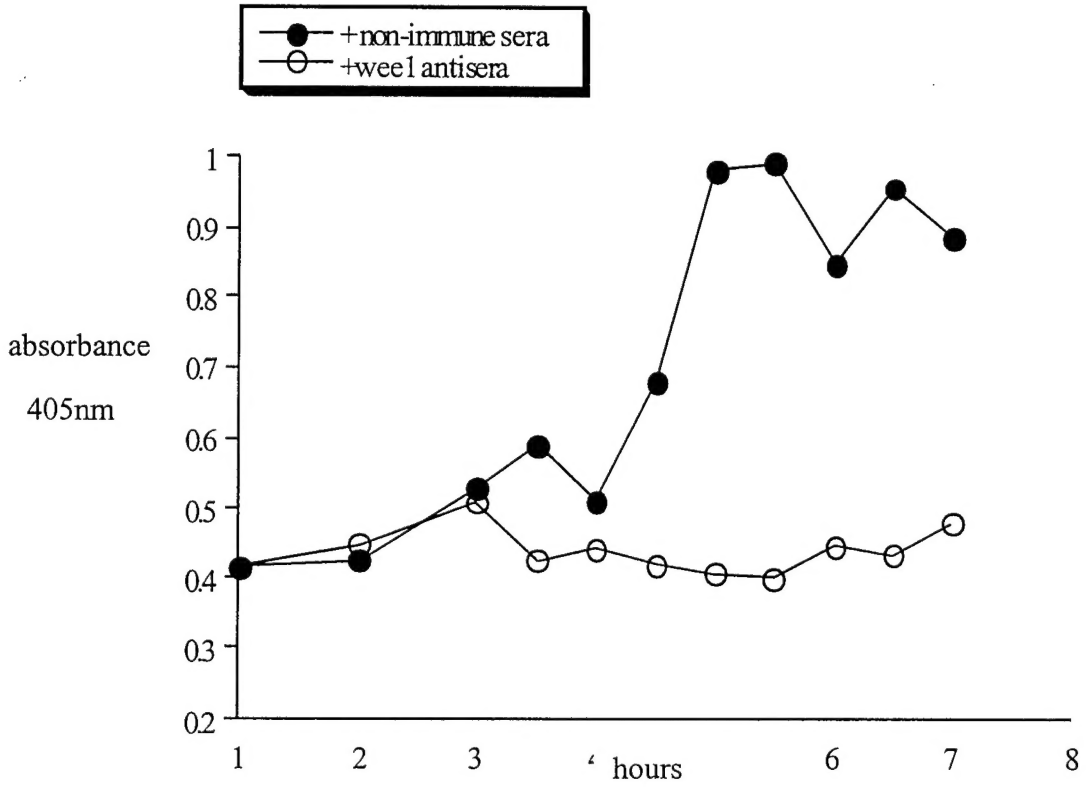

Figure 2. Addition of anti-wee1 antisera to the Xenopus egg extract inhibits apoptosis. Non-immune sera or anti-wee1 antisera was added directly to egg extract at a 1:5 dilution and caspase 3 activity was assayed at 30 minute intervals. The extract supplemented with non-immune sera registered caspase activity at 4.5 hours, however, the extract supplemented with anti-wee1 antisera did not demonstrate any marked increase in caspase 3 activity through 7 hours.

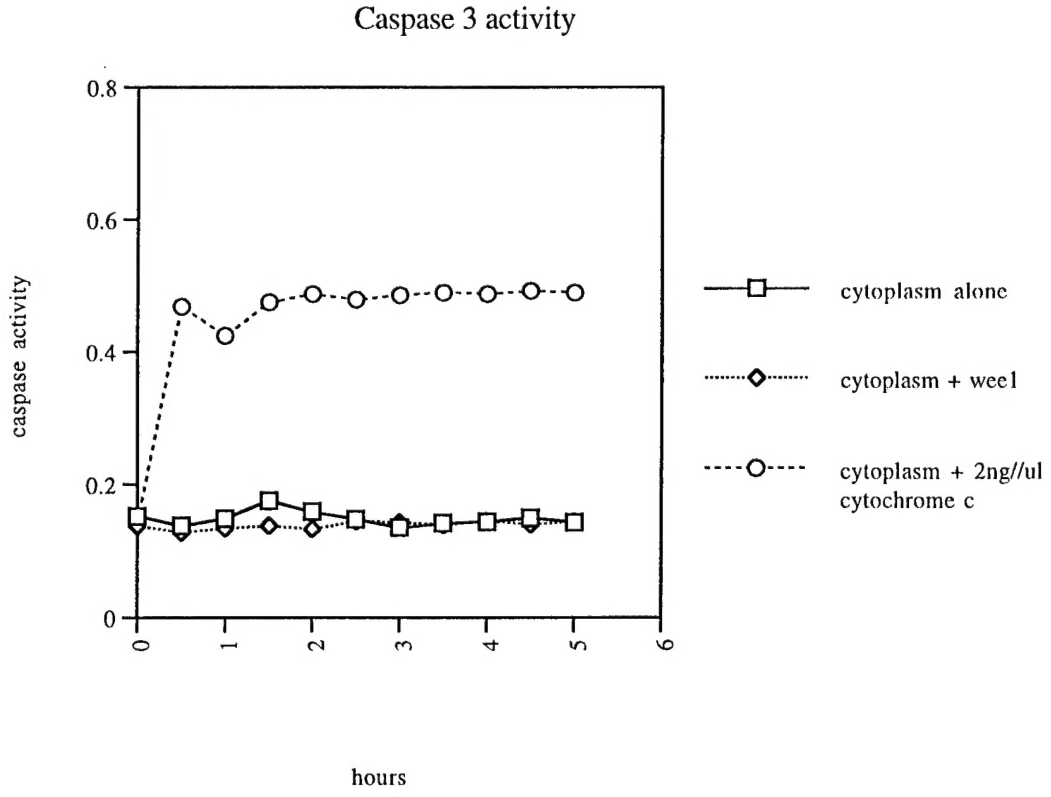

Figure 3. Wee1 acts upstream of mitochondria to accelerate apoptosis in Xenopus egg extracts. Caspase activity was measured in purified cytoplasm, cytoplasm supplemented with wee1, or cytoplasm supplemented with 2ng/ul cytochrome c. As shown above, neither purified cytoplasm alone nor cytoplasm supplemented with wee1 developss caspase activity through 5 hours of room temperature incubation. Purified cytoplasm supplemented with 2 ng/ul cytochrome c was used as a positive control to demonstrate caspase activity could be induced in purified cytoplasm. Caspase activity was detected after only 30 min. room temperature incubation in the cytosol plus cytochrome c sample. The above results demonstrate that wee1 requires the presence of mitochondrial fraction in the extract to accelerate apoptosis.

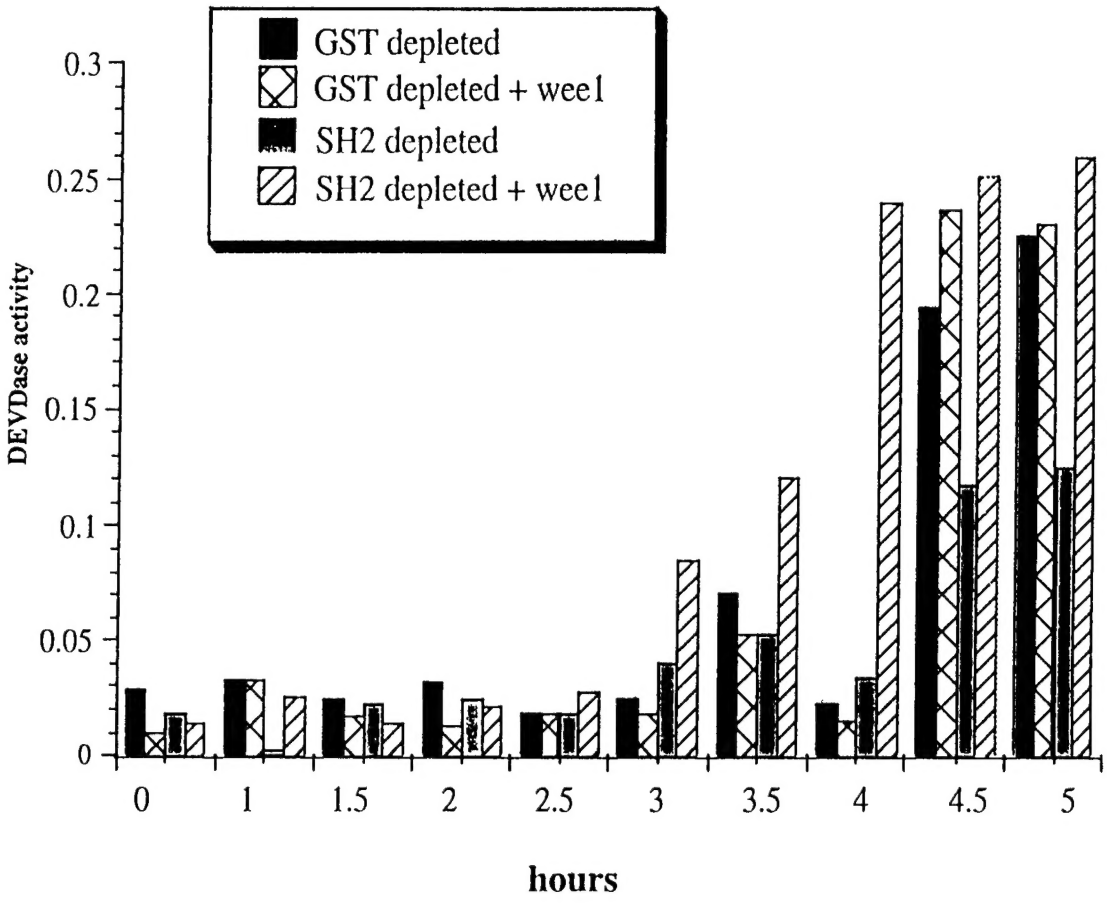

Figure 4. Wee1 rescues apoptosis in a crk SH2 depleted extract. The egg extract was depleted on GST or GST-crk SH2 beads to remove binding proteins. Following depletion, the extracts were supplemented with recombinant wee1 protein or buffer (1:10) dilution and allowed to incubate at room temperature. Apoptosis was assayed by caspase activation. As demonstrated in the 4.5 and 5 hour timepoints, the control GST depleted extrats displayed high caspase activity. The caspase activity in the GST-SH2 dpleted extract remained low, but was restored to control levels upon addition of wee1 protein. Timepoints were assayed for caspase activity in duplicate and averaged. The averaged value for each timepoint is plotted above.

Appendix

Key Research Accomplishments

- Identified crk SH2 ligand in apoptotic egg extracts as the wee1 tyrosine kinase
- Showed that addition of wee1 directly to extracts accelerates apoptosis by 1-1.5 hours
- Showed that addition of wee1 directed polyclonal antisera to extract inhibits apoptosis
- Placed wee1's pro-apoptotic activity upstream of mitochondrial requirement
- Demonstrated that wee1 rescues apoptosis in a crk SH2 ligand depleted extract

Reportable Outcomes

With support of this fellowship, Erica Evans defended her dissertation and received her Ph.D. from Duke University in the fall semester of 1999.